

## U.S. DEPARTMENT OF COMMERCE PATENT &amp; TRADEMARK OFFICE

B/O Form PTO-1390	Transmittal Letter to the United States Designated/Elected Office (DO/EO/US) Concerning a Filing Under 35 USC 371		Attorney's Docket Number REF/29713/Niklasson
International Application Number PCT/SE97/01515	International Filing Date 9 September 1997	U.S. Application Number (if known) <b>09/147801</b>	
Title of Invention New Picornaviruses, Vaccines and Diagnostic Kits		Priority Date Claimed 11 September 1996	
Applicant(s) for DO/EO/US Bo NIKLASSON			

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items under 35 USC 371:

1.  This is a **FIRST** submission of items concerning a filing under 35 USC 371.
2.  This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371.
3.  This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).
4.  A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5.  A copy of the International Application as filed 35 USC 371(c)(2).
  - a.  is transmitted herewith (required only if not transmitted by the International Bureau).
  - b.  has been transmitted by the International Bureau.
  - c.  is not required, as the application was filed in the United States Receiving Office (RO/US).
6.  A translation of the International Application into English (35 USC 371(c)(2)).
7.  Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
  - a.  are transmitted herewith (required only if not transmitted by the International Bureau).
  - b.  have been transmitted by the International Bureau.
  - c.  have not been made; however, the time limit for making such amendments has NOT expired.
  - d.  have not been made and will not be made.
8.  A translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).
9.  An oath or declaration of the inventor(s) (35 USC 371(c)(4)). (  Executed       Unexecuted)
10.  A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).

*Items #1 to 16 below concern other document(s) or information included:*

11.  An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12.  An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13.  A **FIRST** preliminary amendment.  
 A **SECOND** or **SUBSEQUENT** preliminary amendment.
14.  A substitute specification.
15.  A change of power of attorney and/or address letter.
16.  Other items or information:

<i>Application Number (if Known)</i>	<i>International Application Number</i>												
	PCT/SE97/01515												
			<i>Attorney's Docket Number</i>										
			REF/29713/Niklasson										
			<b>Calculations</b>										
			<b>PTO USE ONLY</b>										
<p>17. The following fees are submitted:</p> <p><b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b></p> <table> <tr> <td><input type="checkbox"/> Search report has been prepared by the EPO or JPO . . . . .</td> <td>\$840.00</td> </tr> <tr> <td><input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) . . . . .</td> <td>\$670.00</td> </tr> <tr> <td><input type="checkbox"/> No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but International Search Fee paid to USPTO (37 CFR 1.445(a)(2)) . . . . .</td> <td>\$760.00</td> </tr> <tr> <td><input checked="" type="checkbox"/> Neither International Preliminary Examination Fee (37 CFR 1.482) nor International Search Fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . .</td> <td>\$970.00</td> </tr> <tr> <td><input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . .</td> <td>\$96.00</td> </tr> </table>				<input type="checkbox"/> Search report has been prepared by the EPO or JPO . . . . .	\$840.00	<input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) . . . . .	\$670.00	<input type="checkbox"/> No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but International Search Fee paid to USPTO (37 CFR 1.445(a)(2)) . . . . .	\$760.00	<input checked="" type="checkbox"/> Neither International Preliminary Examination Fee (37 CFR 1.482) nor International Search Fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . .	\$970.00	<input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . .	\$96.00
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<input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . .	\$96.00												
<b>ENTER APPROPRIATE BASIC FEE AMOUNT</b>			\$ <b>970.00</b>										
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).													
<b>CLAIMS</b>	<b>NUMBER FILED</b>	<b>NUMBER</b>	<b>RATE</b>										
Total Claims	14	-20 =	× \$18.00										
Independent Claims	2	-3 =	× \$78.00										
Multiple Dependent Claims (if applicable)			+ \$260.00										
<b>TOTAL OF ABOVE CALCULATIONS</b>			\$ <b>0.00</b>										
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity Statements must also be filed (Note 37 CFR 1.9, 1.27, 1.28)													
<b>SUBTOTAL</b>			\$ <b>970.00</b>										
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).													
<b>TOTAL NATIONAL FEE</b>													
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property.			\$ <b>40.00</b>										
<b>TOTAL FEES ENCLOSED</b>			\$ <b>1,010.00</b>										
		Amount to be:	Refunded: Charged:										

- a.  A check in the amount of **\$ 1,010.00** to cover the fees is enclosed.
- b.  Please charge my **Deposit Account Number 02-0200** in the amount of **\$ \_\_\_\_\_** to cover the above fees.  
A duplicate copy of this sheet is enclosed.
- c.  The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to **Deposit Account Number 02-0200**. A duplicate copy of this sheet is enclosed.

Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

**BACON & THOMAS, PLLC**  
 625 SLATERS LANE - FOURTH FLOOR  
 ALEXANDRIA, VIRGINIA 223124-1176  
 (703) 683-0500

*Respectfully submitted,*

  
**Richard E. Fichter**  
*Attorney for Applicant*  
 Registration Number: 26,382

DATE: March 11, 1999

09/147801

300 Rec'd PCT/TTO 11 MAR 1999

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of : Attention: PCT OFFICE  
Bo NIKLASSON :  
U.S. National Phase of PCT/SE97/01515 :  
Entry papers filed herewith on March 11, 1998 :  
For: NEW PICORNAVIRUSES, VACCINES AND DIAGNOSTIC KITS

PRELIMINARY AMENDMENT  
AND INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner of Patents  
Washington, D.C. 20231

Sir:

Please amend the above-identified application as follows:

IN THE CLAIMS:

Please note that the amended sheets 28, 29, 30 and 31 attached to the International Preliminary Examination Report (Annexes) and submitted herewith, have replaced the originally filed pages 28 - 31 of the application. The claims to be examined and amended by this preliminary amendment are found on **amended sheets 28-31.**

Claim 5, line 2, delete "any one of claims 1-3" and insert --claim 1--.

Claim 6, line 2, delete "any one of claims 1-3" and insert --claim 1--.

Claim 7, line 2, delete "according to claim 5";

line 5, delete "according to any one of claims 1-3";

line 7, after "virus" insert a period -- . --;

delete line 8 in its entirety.

Delete claim 8 without prejudice or disclaimer and insert the following new claim:

--15. Vaccine having as an immunizing or neutralizing component a member selected from the group consisting of  
the virus according to claim 1  
and  
DNA corresponding to the genomic RNA of the virus.--

Claim 10, line 1, delete "any one of the claims 1-3" and insert --claim 1--;

line 2, delete "according to claim 5--;

line 3, delete "according to claim 6".

Claim 11, line 1, delete "any one of the claims 1-3" and insert --claim 1--

line 2, delete "according to";

line 3, delete "claim 5"

delete "according to claim 6,".

Delete claim 13 without prejudice or disclaimer and insert the following new claim:

--16. Method of prophylactic or therapeutic treatment of a disease caused by a virus according to claim 1 in a mammal, including human, which comprises administering to said mammal a prophylactically or therapeutically effective amount of a medicament comprising as an active ingredient a member of the group consisting of the virus according to claim 1,

an antigen including a subunit of the virus,

and

DNA corresponding to the genomic RNA of the virus.--

Please add the attached ABSTRACT OF THE DISCLOSURE to the application.

**REMARKS**

Applicant has amended the application to substitute the originally filed pages 28 - 31 with the **amended sheets** 28 - 31 attached to the International Preliminary Examiner Report (Annexes) and included in the application as filed herewith.

Applicant has amended the claims in order to reduce the filing fee by deleting the multiple dependencies. Applicant retains the right to reintroduce any subject matter canceled by the present Amendment at any time during the prosecution of this application or any continuation or divisional thereof in the United States. Also, an Abstract of the Disclosure has been added to the application.

Applicant respectfully submits a Sequence Listing on computer disk in computer readable format.

Applicant is submitting herewith a copy of the Search Report which issued on International Application No. PCT/SE97/01515, of which the present application is the U.S. national phase. All of the publications cited in the International Search Report are listed on the attached Form PTO-1449. It is Applicants' understanding that, under the procedures of the PCT, copies of the cited publications will have been supplied to the U.S. Patent Office by the International Bureau. However, the Examiner is invited to contact the undersigned attorney if additional copies are necessary or would facilitate examination of the present application.

Otherwise, the Examiner is respectfully requested to return an initialed and dated copy of the attached Form PTO-1449 to confirm that all publications listed thereon have been considered and made officially of record in the file of this application.

Applicant understands that, under the procedures of the PCT, a copy of the priority document (SE 9603305-5, filed 11 September 1996) will have been supplied to the U.S. Patent Office pursuant to Rule 17 of the PCT Regulations. It is therefore

U.S. National Phase of PCT/SE97/01515

respectfully requested that the first Official Action in the present application contain an indication that the appropriate priority document is in the file of this application.

Respectfully submitted,  
BACON & THOMAS, PLLC

By:   
RICHARD E. FICHTER  
Registration No. 26,382

625 Slaters Lane - 4th Floor  
Alexandria, Virginia 22314  
Phone: (703) 683-0500  
Facsimile: (703) 683-1080  
REF:lmh  
Niklasson.PA.wpd

DATE: March 11, 1999

ABSTRACT OF THE DISCLOSURE

A new group of picornaviruses is disclosed. The picornaviruses of the invention comprise in the non-coding region of their viral genome a nucleotide sequence which corresponds to cDNA sequence (I) or homologous sequences having at least 75 % homology to the SEQ ID NO:1, and they cause mammalian disease. Further aspects of the invention comprise a protein corresponding to a protein of the picornaviruses, antiserum or antibody directed against a protein of the picornaviruses, antigen comprising a protein of the picornaviruses, diagnostic kits, vaccines, use of the picornaviruses in medicaments, particularly for the treatment or prevention of Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome, and methods of treatment of diseases caused by the picornaviruses.

**SEQ ID NO: 1 (Ljungan 87-012)**

AGTCTAGTCT TATCTTGAT GTGTCCTGCA CTGAACTTGT TTCTGTCTCT	50
GGAGTGCTCT ACACCTCAGT AGGGGGCTGTA CCCGGGCGGT CCCACTCTTC	100
ACAGGAATCT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCACACC	150
CGCTCCACGG TAGAAGATGA TGTGTGTCTT TGCTTGTGAA AAGCTTGTGA	200
AAATCGTGTG TAGGCGTAGC GGCTACTTGA GTGCCAGCGG ATTACCCCTA	250
GTGGTAACAC TAGC	

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10 Rec'd PCT/PTO 11 1 MAR 1999

NEW PICORNAVIRUSES, VACCINES AND DIAGNOSTIC KITS.

## 5 FIELD OF INVENTION

The present invention relates to new picornaviruses, proteins expressed by the viruses, antisera and antibodies directed against said viruses, antigens comprising structural proteins of said viruses, diagnostic kits, vaccines, use of said viruses, antisera or antibodies and antigens in medicaments, and methods of treating or preventing diseases caused by said viruses, such as Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome.

## 15 BACKGROUND OF THE INVENTION

Recently, a sudden death syndrome among Swedish orienteers has been observed. Of approximately 200 elite orienteers six died in myocarditis during 1989-1992 (1). Orienteering, aiming to find the fastest/shortest way between several checkpoints and often in forested areas, is exceptional with respect to environmental exposure. Thus it has been speculated, that the sudden deaths syndrome among orienteers is caused by a vector borne (rodent or arthropod) infectious agent.

25 It has now been shown in an epidemiological study that the incidence of deaths in myocarditis in northern Sweden tracked the 3-4 year population fluctuations (cycles) of bank voles (*Clethrionomys glareolus*) with one year time lag. Previously, it has been shown that cardioviruses, with rodents as their natural reservoir, can cause Guillain Barré Syndrome (GBS) in man, Diabetes Mellitus (DM) in mice and myocarditis in several species including non-human primates.

In addition to death in myocarditis it is also shown in the epidemiological study that the number of patients diagnosed with Guillain Barré Syndrome (GBS), and Diabetes Mellitus (DM) in northern Sweden tracked the 3-4 year population fluctuations of bank voles with different time delays.

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Sven Gard and co-workers studied antibody prevalence to encephalomyelitis virus (EMCV) in Swedish normal population in the early 1950th (2). These studies found a surprisingly high antibody prevalence rate by hemagglutination inhibition test but no sera could be confirmed by neutralization test. These results were found puzzling at the time but could be explained by the presence of one or several related picornaviruses circulating in Sweden.

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The fact that enterovirus have a large number of members and cardiovirus only two possibly three could reflect the true diversity of the two genus or only be the result of the amount of effort made to isolate new viruses from rodents as compared to isolating new enteroviruses from humans.

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The Picornavirus family is presently divided into five genera (aphto-, entero-, hepato-, rhino-, and cardioviruses) (3). This taxonomy was initially based on morphological, physiological and serological properties as well as on the pathogenicity of the viruses. More recently, however, viruses have been characterized based on their genome sequence since it has been established that sequence data to a large extent coincide with the characterisation properties used previously (4,5).

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The prototype virus in the cardiovirus genus is Theiler's murine encephalomyelitis virus (TMEV). Another member in this genus is encephalomyocarditis virus (EMCV). Vilyuisk virus, isolated from patients in Russia with degenerative neurological disease, is serologically related to TMEV but presently under consideration for being included as a third distinct member of the cardiovirus genus (6).

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In nature, cardioviruses have a geographically widespread distribution and a large number of susceptible hosts with rodents as their natural reservoir. In addition to rodents, EMCV has been isolated from domestic pigs, elephants, lions, non human primates and man (7,8,9). Infection with TMEV and EMCV have provided excellent animal models for inducing myocarditis, DM and different neurological disorders such as demyelinating diseases resembling multiple sclerosis in mice (10-16). Other neurological or muscular disorders in which an infection is suspected to be the triggering factor and in which there is also an autoimmune component are Cardiomyopathy, Multiple Sclerosis (MS), Chronic Fatigue Syndrome (CFS), Myasthenia Gravis (MG), and Amyotrophic Lateral Sclerosis (ALS). It has never been established, however, that cardiovirus is a significant human pathogen, as disease in man most often has been described in case reports or as infection measured in sero-epidemiological surveys (7-17).

Thus, there may be other not yet identified picornaviruses circulating in the wild rodent population and occasionally infecting humans resulting in Myocarditis, Cardiomyopathy, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyotrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome, in genetically susceptible individuals.

The epidemiological link between important human diseases and small rodent abundance and what is previously known about picornavirus/cardiovirus motivated attempts to isolate novel picornaviruses from small rodents.

## DESCRIPTION OF EXPERIMENTAL WORK AS BASIS FOR THE INVENTION

### Trapping of animals

Small rodents were trapped at several locations in northern Sweden and transported live to the Swedish Institute for Infectious Disease Control in Stockholm, Sweden. Species, date and location of trapped animals were

recorded. Animals were bled using ether anaesthesia and killed. Organs were immediately removed and stored at -70°C until tested for presence of virus. A total of 53 *Clethrionomys glareolus* and 28 *Microtus agrestis* were tested for virus isolation.

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### **Virus isolation**

The isolation technique used in the present study was different from what is most often used. The cells used for isolation were kept for a minimum of two weeks and virus growths were detected by both CPE (cytopathogenic effect) and by staining the cells by a large number of human sera using IFT (immunofluorescence test). None of the new viruses presented herein would have been isolated using routine procedure for detecting cardioviruses/picornaviruses. They grow to lower titer and CPE develops slowly.

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Saliva mixed with lung homogenate and faeces were analyzed separately from each animal. The material was inoculated into T25 flask of confluent BHK-21 cells. Cells were blind passed twice a week during two weeks. At the end of this period or earlier if signs of CPE occurred, cells were removed from the T25 flask by a rubber policeman, placed onto 10-well spot slides, air dried and acetone fixed. The cells were then stained with panels of human sera including 5 multiple sclerosis patients, 5 patients recently diagnosed with DM and 5 athletes dying in myocarditis and bled at autopsy. All T25 flasks (saliva-lung and faeces separately) were tested individually by IFT using the complete panel of human sera at a 1:10 dilution.

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Cells showing positive reaction by IFT using the human serum panels were selected for further analysis. This included inoculation intracerebrally into 1 day old suckling mice, serological characterisation and sequence analysis.

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### **Antisera and serological procedures**

Antisera to the virus isolates were raised in mice (NMRI), and Guinea Pigs (Dunkin Hartley). The animals were injected with a cell culture supernatant from

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(BHK-21 cells) intraperitoneally and serum collected 4-6 weeks later.

Preimmunization sera were tested individually while postimmunization sera were pooled from all infected animals.

- 5 An indirect immunofluorescence test (IFT), as described previously (18,19) was used to test antibody titres in immunized animals. Briefly, spot slides were prepared by incubating virus on Green Monkey Kidney (GMK) cells for 6-10 days. At sign of discrete CPE cells were removed from the flask by a rubber policeman and put onto the microscope slides, air dried, and fixed in cold (4°C) acetone and stored at -70°C. The titer was determined by incubating serum diluted in PBS in the slides at 37°C for 1 hour in a moist chamber, followed by a FITC conjugate (F(ab')<sub>2</sub>) fragment of goat anti human IgG γ-chain specific, Sigma Immuno Chemicals (F-1641) or Rabbit anti mouse immunoglobulins Daco (F0313) incubated as above.
- 10
- 15 Antibody titers to the viruses were determined by a modification of the Plaque reduction neutralization test (PRNT) as described by Earley et al (20). In the test, sera were serially diluted four-fold and mixed with an equal volume containing 80-100 plaque-forming units (pfu) of virus per 50 µl. The mixtures were then incubated at 37°C for 60 minutes, and 50 µl subsequently inoculated into each of 2 wells of a tissue culture plate containing confluent Vero cell monolayer. After adsorption for 60 minutes at 37°C the wells were overlaid with 0.5 ml of a 42°C mixture of 1 part 1% agarose and 1 part 2X basal Eagle's medium with Earle's salts, 17 mM Hepes buffer, 8% heated fetal calf serum, 100 U/ml penicillin, 100 µg streptomycin. The tissue culture plates were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 3-7 days. A second overlay (0.5 ml) containing neutral red stain (1:9000) was then applied and plaques were enumerated the following day. The plaque numbers were linearly extrapolated to 2-fold dilutions. A 50% reduction of plaques was used as the criterion for virus neutralization titers.
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### Electron microscopy

Cell culture media or brain tissue homogenates were examined by negative contrast electron microscopy (EM). A 10 µl droplet was incubated on Formvar/carbon-coated grids for one minute or alternatively, 0.5 ml samples were centrifuged for 30 minutes at 20,000 x g to remove cell debris and finally the supernatants were pelleted directly onto grids in a Beckman Airfuge for 10 minutes at 160,000 x g. Grids were stained with 2% phosphotungstate acid (pH 6.0) and examined in a Philips CM 100 electron microscope at a magnification of at least 46,000.

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### Sequence data

The isolates 87-012, 174F and 145SL were grown on the human lung carcinoma line A549 in 1600 cm<sup>2</sup> roller bottles. Full CPE was observed after 5-10 days. Supernate was filtered through 0.45 µM cellulose acetate filters (Costar) and the virus was pelleted at 20,000 g for 20 h at 4°C. RNA was isolated from the virus containing pellets using acid guanidinium thiocyanate as described (Chomczynski and Sacchi). Synthesis of cDNA was performed under standard conditions using 1 µg of RNA, AMV reverse transcriptase (Boehringer-Mannheim) and random 14 mer oligonucleotides as primers in a 20 µL reaction. Fragments of the viral 5'UTR were amplified using cardiovirus specific consensus primers: (sense) 5'-GGCCGAAGCCGCTTCCAATA-3' (SEM) and (antisense) 5'-GTGGCTTTGCCGCAGAG-3' (ATVEM), both primers modified after the EMCV2 and EMCV1 primers previously reported (Jongen et al. 1993. Ann. Reum. Dis. 52:575-578. Cardiovirus sequences were from Dr A. Palmenberg (personal communication). Amplification conditions were 30 cycles at: 94°C, 30 sec., 50°C, 30 sec, 72°C, 2 min. The amplified fragments were cloned into the pCRII T-vector (In-Vitrogen). The cloned viral sequences were sequenced using A Taq polymerase FS cycle sequencing kit and data was collected on a ABI Prism 310 sequencing machine using M13 -21 and M13 reverse primers (Perkin-Elmer). A 1.8 kb fragment extending from the 5'-UTR into the viral polyprotein sequences was obtained by PCR (polymerase chain reaction) amplification of cDNA from the 145SL isolate. The primers were:

(sense) 5'-ACAGTGCATTCCACAC-3' (SLJU1) or 5'-CCGCTCCACAATAGA-3' (SLJU2) and (antisense) 5'-GATCTCAGAC-3' (primer 118). The SLJU1 and SLJU2 primers are located immediately adjacent to one another and were chosen as consensus primers for the Ljungan isolates of the invention with as little homology as possible to the EMCV and TMEV groups of viruses. The amplification conditions were 30 cycles at: 94°C, 30 sec., 42°C, 1 min , 72°C 2 min. The antisense primer 118 yielded similarly sized PCR products with either the SLJU1 or SLJU2 as sense primers, but none of the primers yielded PCR fragments when used alone. The sequence of the primer 118 was previously published (Bauer, D., et al. 1993. Nucl. Acids Res. 21:4272-4280). The obtained 1.8 kb PCR fragment was cloned and sequenced as described above.

## RESULTS OF EXPERIMENTAL WORK

Three virus isolates were selected based on reaction with the human serum panels and showing a size and structure compatible with a picornavirus on EM. The first isolate was named Ljungan 87-012. Ljungan is a river in Medelpad county, Sweden where the animals were trapped.

The second and third isolate were designated Ljungan 174F and Ljungan 145SL, respectively.

All three isolates came from *C. glareolus*.

All three isolates killed suckling mice in 3-5 days.

The titer in mouse brain was 10<sup>9</sup> (approximately) while the cell culture titer was only 10<sup>5</sup> (approximately).

## 25 Electron microscopy

Virus particles, 27 nm in diameter, were spherical with the surface almost featureless and they appeared single or in small aggregates. In rare cases the stain penetrated the particles which made them look like empty shells.

**Serological results**

It was found after testing a number of different cell lines the Green Monkey Kidney cells were most suitable for making IFT drop slides for serology. The cross IFT data using mouse sera are seen in Table 1.

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**TABLE 1**

Cross-IFT using virus infected GMK cells. Immune mice were titrated using 4 fold dilutions starting at a 1:10 dilution.

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**VIRUS**

87-012 174F 145SL

**Antisera**

87-012	<b>2560</b>	160	<10
174F	160	<b>160</b>	<10
145SL	40	40	<b>640</b>

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PRNT (plaque reduction neutralization test) data, preliminary results. Rabbit sera against TEMV and EMCV with a titer of 1:160 homologous had a titer less than 10 to the three isolates. Several attempts to make antisera with neutralizing titer in bank voles, mice, rabbits and guinea pigs have failed. All animals made high titer antibodies by IFT but no by PRNT. Bank voles failed to make IFT antibodies.

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**Sequence data**

Sequences from 5'UTR and polyprotein gene of Ljungan virus isolates.

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Cardiovirus consensus primers yielded a product of 303 bp for the three isolates 87-012, 174F and 145SL, compared to 284 bp for EMC virus. The fragment amplified was located immediately after the end of the poly C tract in EMC virus. PCR products specific for the Ljungan isolates were only obtained when the reannealing temperature was 50°C, and not at 58°C, which was optimal for

obtaining products from EMC virus cDNA. The subsequent sequence analysis revealed that the ATVEM primer was mismatched at 4 internal positions, explaining this difference in reannealing temperature. An alignment of the 5'UTR sequences for the three Ljungan isolates, EMCV and Vilyuisk virus (Table 2) shows a greater similarity between EMCV and Vilyuisk virus than between either of the two and the Ljungan isolates. It also demonstrates that each Ljungan isolate is distinct from the other by a number of nucleotide changes. The 174F and 145SL are similar to the isolate 87-012. The sequence homology between 174F and 87-012 was at most 95% (three undetermined bases in the sequence) while the homology between 87-012 and 145SL was 91%.

The strategy chosen for obtaining additional PCR fragments from the Ljungan virus isolates was a modification of a technique for detecting differentially expressed mRNAs (Bauer, D., et al. 1993. Nucl. Acids Res. 21:4272-4280). As a test for this strategy, cDNA from the Ljungan 145SL isolate was amplified using the conditions above, using either the SLJU1 or the SLJU2 primer as a sense primer and one of twenty 10-mer oligonucleotides of randomly chosen sequence as "antisense" primer.

If the PCR products obtained with the SLJU1 or SLJU2 primers and a specific 10-mer were similarly sized, and none of the primers yielded a product of this size when used alone in the PCR reaction, the fragment obtained was isolated and cloned. Only one combination of primers satisfied this criterion, namely the SLJU1 or SLJU2 primers in combination with the 118 10-mer oligonucleotide, which yielded a 1.8-1.9 kb PCR product. Of this fragment, 819 bp were sequenced from the 3' end. This sequence contained an open reading frame (ORF) of 663 bp in the sense of the viral polyprotein. This ORF was used to search in the Swiss protein data bank using the BLITZ search service from EMBL with the default search parameters. The top 10 scores were picornavirus polyprotein sequences, including 8 cardiovirus sequences. Homology was found over 188 a.a. The relatedness of this segment of the viral polyprotein to previously sequenced cardioviruses is shown in Table 3. A comparative

alignment of all cardioviruses was made available to us by Dr. A. Palmenberg. In Table 3, the sequence of TMEBeAn was arbitrarily taken as the index strain. For the 12 remaining cardioviruses in the alignment, only differences in amino acid sequence are shown. The alignment of the Ljungan 145SL sequence is similarly represented at the top. Since the BLITZ search algorithm takes into account identical as well as similar amino acids, the latter have been indicated by small type, while differences to TMEBeAn is in capitals as for the other strains in the alignment.

In conclusion, the above presented data for the Ljungan isolates are characteristic for the 3 viruses but yet incomplete. However, the comparison of cloned sequences from both a highly conserved part of the 5'-untranslated region of cardioviruses and coding sequences for the viral capsid proteins of one isolate (Ljungan 145SL) clearly show that the Ljungan viruses are related to the cardioviruses, but are more distant relatives than any previously identified cardiovirus. While the amino acid homology (identical amino acids) of the viruses within the Theiler group is 96-97%, the homology to Vilyuisk virus is about 83%, and the EMC viruses are 67-74% homologous to TMEBeAn, the Ljungan 145SL has only about 32% identical amino acids to TMEBeAn. Even if homology is taken as identical and similar amino acids, this measure of relationship would still amount to only 50% between Ljungan 145SL and TMEBeAn (the corresponding figure would be 79-83% between EMC and TMEBeAn).

## ALIGNMENT OF SEQUENCES

Table 2 shows an alignment of three Ljungan virus isolates (1. 87-012, 2. 174F, 3. 145SL)[SEQ ID NO: 1,2 and 3, respectively] with published cardiovirus sequences (4. TMEBeAn, 5. Vilyuisk, 6. EMCV). The aligned sequence starts 29 nt 3' of the end of the poly-C tract in EMCV, and the sequence corresponds to nt 557 - 808 (approximately) in the different viral genomes. Inserted spaces in the sequences are indicated by a period (.).

**TABLE 2**

1. AGTCTAGTCTTATCTTGTATGTGTCCTGCACT..GA..ACTTGTTCTGT  
 5 2. AGTCTAGTTCATCTGTGTGTTGGCACT..GA..AATTATTCCTGT  
 3. AGTTGGTTCTCTCTTGAGTGTGTTTGTT..AG..CATAATTCTGT  
 4. TGACAGG.GTTATTTCACCC.TCTTCTT..TTCTACTCCACAG.TG.T.T  
 5. TGACAGG.GTTATTTCACCC.TCTTCTCTTCTACTTCATAG.TG.T.T  
 6. AGGCCGGTGTGCGTTGTCTATATGTTATTTCCAC..CATA.TTGCCGT
- 10 1. CTCTGGAGTGCTCTACACTTCAGTAGGGGCTGT.A.CCCGGGCGGTCCCC  
 2. CTCTGGGGTGCTTACACTTCAGTAGGGGCTGT.A.CCCGGGCGGTCCCC  
 3. CTCTAGAGTGCTTACACTCTAGTAGGGGCTGT.A.CCCGGGCGGTCCCC  
 4. CT.A.....TACTGTG..GAAGGGTATGTGT....TGCCCCTTCCT  
 15 5. CT.A.....TACTATG.AA.AGGGTATGTGT..C..GCCCTTCCT  
 6. CT.T.....TTGGCAATGT.G.AGGGCCCG.GAAACCTGGCCCTGTCT
- 20 1. CTCTTCACAGGAATCTGCACAGGTGGCTTCAC.CTCTGGACAGTGCATT  
 2. CTCTTCACAGGAATNTGCACAGGTGGCTTCAC.CTCTGGACAGTGCATT  
 3. CTCTTCACAGGAATCTGCACAGGTGGCTTCAC.CTCTGGACAGTGCATT  
 4. .TCTTGGAGAACGT..GCGCGGCGGTCTTCCGTCTCGACAA.GCGC.  
 5. .TCTTGGAGAACGT..GCGTGGCGGTCTTCCGTCTCGAAAAACG..T  
 6. .TCTTGACGAGCAT.T.CCTAGGGTCTTCCC.CTCTGCCAAAGGAAT
- 25 1. CCACACCCG.C.TCCACGGTAGAAGATGATGTGTCTTGCT..TGTGA  
 2. CCACACCCG.C.TCCACAGTAGAAGATGATGTGTCTTGCT..TGTGA  
 3. CCATACCCG.C.TCCACAATAGAAGATGATGTATATCTTGTT..TGTGA  
 4. GCGT..GCAACATACAGAGT.AACG.CGAAGAA.AGCA..GTTC.TC.GG  
 5. GCGT..GCGACATGCAGAGT.AACG.CAAAGAA.AGCA..GTTC.T.TGG  
 30 6. GCA.A.G.GTC.TGTTGAAT.GTCG.TGAAGGA.AGCA..GTTCTCTGG
- 35 1. AAA.GCTT...GTGAAAATC.....GTGTGTAGGCGTAGCGGCTACT  
 2. AAA.GCTT...GTGAAAATC.....GTGTGTAGGCGTAGCGGNTACT  
 3. AAT.GCT.CA..TGAA.A.C.....GTGTGTAGGCGTAGCGGCTACT  
 4. TCTAGCT.CTAGTGCCCA.CAAGAAAACAGCTGTAG.CG.ACCA.C.ACA  
 5. TCTAGCT.CTGGTGCCCA.CAAGAAAACAGCTGTAG.CG.ACCA.C.ACA  
 6. AA..GCTTCT..TGAAGA.CAA.ACAACGTCTGTAG.CG.ACC..CT..T
- 40 1. TGAGTGCCAGCGGATTACCCCTAGTGGTAACACTAGC  
 2. TGAGTGCCAGCGGACNACCCCTAGTGGTAACACTAGC  
 3. TGAATGCCAGCGGAACCCCCCTAGTGGTAACACTAGC  
 4. ..AAGGC.AGCAGGAACCCCCCTCCTGGTAACAGGAGC  
 5. ..AAGGC.AGCAGGAACCCCCCTCCTGGTAACAGGAGC  
 6. TGCAGGGC.AGCAGGAACCCCCCACCTGGCGACAGGTGC
- 45

In this region of the viral genome, Ljungan 174F has 94% homology to Ljungan 87-012 (here taken as the indicator strain for comparisons), and Ljungan 145SL

has 91% homologous residues to Ljungan 87-012. The TMEBeAn strain has 69%, Vilyuisk has 68% and EMCV has 68% homologous residues to Ljungan 87-012. Using the same criteria for calculating the homology, EMCV has 85% homology to TMEBeAn.

5

Table 3 shows alignment of cDNA sequences from the polyprotein coding sequences of the Ljungan 145SL isolate [SEQ ID NO. 4] to the amino acid sequences of sequenced cardioviruses in the comparative alignment compiled by Dr. A. Palmenberg (personal comm.) The TMEBeAn strain was arbitrarily taken as the indicator strain, while the amino acids of the remaining strains are shown only if they differ from the indicator strain. For the Ljungan 145SL isolate, similar, but non-identical amino acids are indicated in small type. The amino acid homology between Ljungan 145SL and other cardioviruses was established screening the entire Swiss Protein Data Bank using the BLITZ search algorithm with standard search parameters.

EGB 1500 1998 24 11 50

TABLE 3

Ljungan 464	480	525
145SL	K--m-iArm-sVyK-ERTEPGGTNG--QwshtHSPInW-.fDGGIHLED-P-.-LFsSCy-	
TMEBeAn	SDLLELCKLPT.FLGNPNTNNKRYPYFSATNSVPATSMVDYQVALSCSCMANSMIAVARNFN	
TMEGd7	-----S-D-----L-----	
TMEGd7	-----S-D-----L-----	
TMEDA	-----S-----T-L-----C-----	
Vilyuisk T	-----L.S-DT-V-F-T-----TE-L-E---T-----S-----S-----	
EMCBd	K-F--IAQI--.I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L-TF--LS--A	
EMCBC	K-F--IAQI--.I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L-TF--LS--A	
EMCDd	K-F--IAQI--.I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L-TF--LS--A	
EMCDC	K-F--IAQI--.I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L-TF--LS--A	
EMCDv1	K-F--IAQI--.I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L-TF--LS--A	
EMCR	K-F--IAQI--.I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L-TF--LS--A	
MengoM	K-F--IAQI--.I--KMP-AVP-IEA-N-.A-KTQPLAV---T-----L-TF--LS--A	
Mengo37a	K-F--IAQI--.I--KVP-AVP-IEA-N-.A-KTQPLAV---T-----L-TF--LS--A	

Ljungan 526	540	588
145SL	Yw---TVLKLTVYAstFN--rLRm-fF-I.MMqG-Q-.kKHkCLfMvC-i---nt-EM-I-y.	
TMEBeAn	QYRGSLNLFVFTGAAMVKGKFLIAYTPPGAGKPTTRDQAMQSTYAIWDLGLNSSFNFATPI	
TMEGd7	-----R-----A-----	
TMEGd7b	-----A-----	
TMEDA	-----A-----V-----	
Vilyuisk	-----S-T-----X-----V-----	
EMCBd	VYT-----T-M-----S-A-----YS-V-----	
EMCBC	VYT-----T-M-----S-A-----YS-V-----	
EMCDd	VYT-----T-M-----S-A-----YS-V-----	
EMCDC	VYT-----T-M-----S-A-----YS-V-----	
EMCDv1	VYT-----T-M-----S-A-----YS-V-----	
EMCR	VYT-----T-M-----S-A-----YS-V-----	
MengoM	VYT-----T-M-----S-A-----YS-V-----	
Mengo37a	VYT-----T-M-----S-A-----YS-V-----	

Ljungan 589	600	651
145SL	...-w..GnwMR--RG--I--1RiDV-NR--N-Ss-NAVnCiLQ-KM-n-AKFMv-TT-NIV-	
TMEBeAn	SPTHYRQTSYTSPTITSVDGWVTWKLTPLYPSGPTNSDILTLVSAGDDFTLRMP.ISPTKW	
TMEGd7	-----Q-----	
TMEGd7b	-----Q-----	
TMEDA	-----A-A-----Q-----A-V-----	
Vilyuisk	--S-----S-AA-----L-----Q-----F-ANV-PS-----N-----	
EMCBd	F-MVGTDQVN-N-----Q-----P-C-SAK-----M-----K-S-K--.AP-	
EMCBC	F-MVGTDQ-----Q-----	
EMCDd	F-MVGTDQ-----Q-----	
EMCDC	F-MVGTDQ-----Q-----	
EMCDv1	F-MVGTDQ-----Q-----	
EMCR	F-MVGTDQ-----A-----Q-----	
MengoM	F-MVGTDQA-----Q-----	
Mengo37a	F-MVGTDL-----A-----Q-----	

5           **Serological assay indicating relationship between the Ljungan viruses and diabetes mellitus and myocarditis.**

A serological assay using indirect immunofluorescence test using virus infected acetone fixed green monkey kidney cells was established. Patient sera were screened at a 1:8 dilution and positive sera titrated. Sera with a titer of 1:32 or more were considered positive.

10           Sera from 59 children (age 1-16) from the Stockholm area recently diagnosed with Diabetes Mellitus (DM) and 34 control children from the same geographic area, were tested for presence of antibodies to the three viruses of the invention.

15           Nineteen of the 59 (32%) DM patients screened positive and 2 of the 34 (6%) controls were found positive to one or more of the 3 viruses (significant difference p=0.002, Fisher's exact test). Nine recently diagnosed DM patients (age 23-46) from Medelpad county were also tested. Two controls were selected for each adult DM patient and they were matched for age, sex and geographic area of residence. Five of the nine (56%) DM patients and one of the 18 (6%) control patients were found positive to one or more of the 3 viruses (significant difference p=0.008 Fisher's exact test).

20           Serum was also available from 5 athletes dying suddenly in myocarditis. Three controls were selected for each myocarditis patient and they were matched for age, sex and geographic area of residence. Four of the 5 (80%) patients dying from myocarditis and 1 of the 15 (7%) controls where found positive to one or more of the three Ljungan viruses (significant difference p=0.005, Fisher's exact test).

25           30           **DESCRIPTION OF DIFFERENT ASPECTS OF THE INVENTION**

In the following, different aspects of the invention will be disclosed. However, all of these aspects are related to a new group of picornaviruses.

Thus, a first aspect of the invention is directed to a new group of picornaviruses, namely picornaviruses comprising in their viral genome, more precisely in the non-coding region, a nucleotide sequence corresponding to a cDNA sequence selected from the group consisting of

SEQ ID NO: 1 (Ljungan 87-012)

AGTCTAGTCT TATCTTGTAT GTGTCCTGCA CTGAACTTGT TTCTGTCTCT 50  
GGAGTGCTCT ACACCTCAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC 100  
ACAGGAATCT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCACACC 150  
CGCTCCACGG TAGAAGATGA TGTGTGTCTT TGCTTGTGAA AAGCTTGTGA 200  
AAATCGTGTG TAGGCGTAGC GGCTACTTGA GTGCCAGCGG ATTACCCCTA 250  
GTGGTAACAC TAGC

and homologous sequences having at least 75 % homology to the  
SEQ ID NO: 1. The picornaviruses of the invention should further cause  
mammalian disease.

In a preferred embodiment of this aspect of the invention said homologous  
sequences have at least 80%, at least 85% or at least 90% homology to the  
SEQ ID NO: 1.

In a particularly preferred embodiment said homologous sequence is one of  
SEQ ID NO: 2 (Ljungan 174F)

AGTCTAGTTT CATTCTGTGT GTGTTGGCA CTGAAATTAT TTCTGTCTCT 50  
GGGGTGCTTT ACACCTCAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC 100  
ACAGGAATNT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCACACC 150  
CGCTCCACAG TAGAAGATGA TGTGTGTCTT TGCTTGTGAA AAGCTTGTGA 200  
AAATCGTGTG TAGGCGTAGC GGNTACTTGA GTGCCAGCGG ACNACCCCTA 250  
GTGGTAACAC TAGC

and

SEQ ID NO:3 (Ljungan 145SL).

AGTTGGTTC TCTCTTGAGT GTGTTTGTG TTAGCATAAT TTCTGTCTCT 50  
5 AGAGTGCTTT ACACCTCTAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC 100  
ACAGGAATCT GCACAGGTGG CTTCACCTC TGGACAGTGC ATTCCATACC 150  
CGCTCCACAA TAGAAGATGA TGTATATCTT TGTTTGAA ATGCTCATGA 200  
AACGTGTGTG TAGGCGTAGC GGCTACTTGA ATGCCAGCGG AACCCCCCTA 250  
GTGGTAACAC TAGC.

10

These sequences (ID NO: 2 and 3) have 94% homology and 91% homology to the SEQ ID NO: 1, respectively.

It should be understood that homologies in the coding region of different viruses of the invention may vary considerably, but in the non-coding region they share a homology of at least 75% with the SEQ ID NO: 1.

The nucleotide sequences, SEQ ID NO: 1, 2 and 3, correspond to approximately nucleotides 557 - 808 (a conserved region) in the genome of encephalomyelitis virus (EMCV). These three viruses have been isolated from wild rodents, more precisely bank voles. The viruses can be multiplied in cell lines, and for a large-scale production of picornavirus products the virus genome can be inserted into other microorganisms.

20

25

A second aspect of the invention is directed to a protein comprising an amino acid sequence selected from the group consisting of

SEQ ID NO: 4 ( partial structural protein of Ljungan 145)

Lys Asp Leu Met Glu Ile Ala Arg Met Pro Ser Val Tyr Lys Gly Glu  
5 10 15  
Arg Thr Glu Pro Gly Gly Thr Asn Gly Tyr Phe Gln Trp Ser His Thr  
20 25 30  
5 His Ser Pro Ile Asn Trp Val Phe Asp Gly Gly Ile His Leu Glu Asp  
35 40 45  
Met Pro Asn Leu Asn Leu Phe Ser Ser Cys Tyr Asn Tyr Trp Arg Gly  
50 55 60  
Ser Thr Val Leu Lys Leu Thr Val Tyr Ala Ser Thr Phe Asn Lys Gly  
10 65 70 75 80  
Arg Leu Arg Met Ala Phe Phe Pro Ile Met Met Gln Gly Thr Gln Arg  
85 90 95  
Lys Lys His Lys Cys Leu Phe Met Val Cys Asp Ile Gly Leu Asn Asn  
100 105 110  
15 Thr Phe Glu Met Thr Ile Pro Tyr Thr Trp Gly Asn Trp Met Arg Pro  
115 120 125  
Thr Arg Gly Ser Val Ile Gly Trp Leu Arg Ile Asp Val Leu Asn Arg  
130 135 140  
Leu Thr Tyr Asn Ser Ser Pro Asn Ala Val Asn Cys Ile Leu Gln  
20 145 150 155 160  
Val Lys Met Gly Asn Asp Ala Lys Phe Met Val Pro Thr Thr Ser Asn  
165 170 175  
Ile Val Trp ,  
and homologous sequences having at least 75% homology to the  
25 SEQ ID NO: 4,  
and antigenic fragments of the sequences.  
In an embodiment of the invention the homologous sequences have at least  
80%, at least 85% or at least 90% homology to the SEQ ID NO: 4.

30 The SEQ ID NO: 4 is the result of preliminary partial sequencing of the cDNA sequence from the polyprotein coding sequence of the virus Ljungan 145 SL isolate. Said protein comprising said amino acid sequence SEQ ID NO: 4, said homologous sequences and said antigenic fragments are useful as active ingredients in medicines and as diagnostic reagents in diagnostic kits.

A third aspect of the invention concerns an antiserum or antibody directed against a structural protein of the virus defined in the first aspect of the invention. An example of such a structural protein is defined in the second aspect of the invention. Such an antiserum or antibody is useful as an active ingredient in medicines and as diagnostic reagent in diagnostic kits. Both polyclonal and monoclonal antibodies may be used, and these are suitably produced by using said virus or fragments thereof specific for said virus for immunizing mammals.

10 A fourth aspect of the invention is directed to an antigen comprising at least a part of a structural protein of the picornavirus defined in the first aspect of the invention, including a subunit thereof. An example of such an antigen is the protein and antigenic parts thereof defined in the second aspect of the invention. Such an antigen of the invention is useful as an active ingredient in medicines and as a diagnostic reagent in diagnostic kits.

15 A fifth aspect of the invention is directed to a diagnostic kit comprising at least one member from the group consisting of  
a) an antiserum or antibody according to the third aspect of the invention or an antigen-binding part thereof,  
20 b) an antigen according to the fourth aspect of the invention or an antibody-binding part thereof,  
c) one or several probes designed with respect to the genome of the virus according to the first aspect of the invention,  
25 and  
d) one or several primers designed with respect to the genome of the virus according to the first aspect of the invention.

30 The different members of a diagnostic kit will depend on the actual diagnostic method to be used. In addition to the above-listed possible members of the diagnostic kit, said kit may contain positive reference samples, negative

reference samples, diluents, washing solutions and buffers as appropriate. The kit will further be accompanied by instructions for use.

The above-listed members a) and b) find use in immunodiagnostic methods,  
5 such as enzyme-like immunosorbent assay (ELISA), radioimmunoassay (RIA)  
or immunofluorescence assay (IFA).

The above-listed members c) and d) find use in direct virus detection.

Preferably, a diagnostic method based on the PCR (polymer chain reaction)  
10 technique with such primers is utilized in the direct detection of a virus according  
to the invention.

All of the above mentioned diagnostic methods are well known in the art, and a  
15 man of ordinary skill in the art will readily select useful members for a diagnostic  
kit in relation to the diagnostic method to be used.

A sixth aspect of the invention relates to a vaccine having as an immunizing or  
neutralizing component a member selected from the group consisting of

a) the virus according to the first aspect of the invention,

b) the virus according to the first aspect of the invention in attenuated form,

c) the virus according to the first aspect of the invention in killed form,

d) an antigen according to the fourth aspect of the invention, including a subunit  
of the virus according to the first aspect of the invention,  
and

e) DNA corresponding to the genomic RNA of the virus according to the first  
aspect of the invention.

In an embodiment of this aspect of the invention said vaccine may additionally  
comprises an adjuvant. Such an adjuvant must of course be an adjuvant which  
is approved for use in vaccines by authorities responsible for veterinary or

30 human medicines.

The vaccine may contain other ingredients which are needed for specific preparations intended for oral, subcutaneous, intramuscular or intradermal administration. Suitable additional ingredients are disclosed in the European or US Pharmacopoeia.

5

The alternative members a), b) and c) are all examples of conventional whole virus, attenuated virus, and subunit vaccines developed for other types of viruses, and the member d) represents DNA incorporation into body-specific cells, which then will express virus-specific structures and elicit immunity against said virus.

10

A seventh aspect of the invention is directed to a picornavirus according to the first aspect of the invention, optionally in attenuated or killed form, an antiserum or antibody according to the third aspect of the invention or an antigen according to the fourth aspect of the invention, for use in a medicament (for veterinary or human use). An example of such a medicament is a vaccine according to the invention disclosed in the sixth aspect thereof.

15

The eight aspect of the invention concerns use of a picornavirus according to the first aspect of the invention, optionally in attenuated or killed form, an antiserum or antibody according to the third aspect of the invention or an antigen according to the fourth aspect of the invention, in the preparation of a medicament for prophylactic or therapeutic treatment of a disease caused by said virus.

20

In an embodiment of said use the disease caused by said virus is one of Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome.

25

30

A ninth aspect of the invention is directed to a method of prophylactic or therapeutic treatment of a disease caused by a virus according to the first aspect of the invention in a mammal, including human, which comprises administering to said mammal a prophylactically or therapeutically effective amount of a medicament comprising as an active ingredient a member of the group consisting of

- 5 a) the virus according to the first aspect of the invention,
  - b) the virus according to the first aspect of the invention in attenuated form,
  - c) the virus according to the first aspect of the invention in killed form,
  - 10 d) an antigen according to the fourth aspect of the invention, including a subunit of the virus according to the first aspect of the invention,
- and
- e) DNA corresponding to the genomic RNA of the virus according to the first aspect of the invention.

15 In an embodiment of said method the disease caused by said virus is one of Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome.

20 The actual dosage regimen will be determined by the vaccine producer based on animal experiments and clinical trials.

65 45 62 63 40 32 44 76 30

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: BO NIKLASSON
- (B) STREET: Sibyllegatan 15
- (C) CITY: Stockholm
- (E) COUNTRY: Sweden
- (F) POSTAL CODE (ZIP): 114 42

(ii) TITLE OF INVENTION: NEW PICORNAVIRUSES, VACCINES AND DIAGNOSTIC KITS.

(iii) NUMBER OF SEQUENCES: 4

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 264 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: PICORNAVIRIDAE
- (C) INDIVIDUAL ISOLATE: LJUNGAN 87-012

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGTCTAGTCT TATCTTGTAT GTGTCCTGCA CTGAACTTGT TTCTGTCTCT GGAGTGCTCT	60
ACACATTCAAGT AGGGGGCTGTA CCCGGGCGGT CCCACTCTTC ACAGGAATCT GCACAGGTGG	120
CTTTCACCTC TGGACAGTGC ATTCCACACC CGCTCCACGG TAGAAGATGA TGTGTGTCTT	180
TGCTTGTGAA AAGCTTGTGA AAATCGTGTG TAGGCGTAGC GGCTACTTGA GTGCCAGCGG	240
ATTACCCCTA GTGGTAACAC TAGC	264

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 261 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Picornaviridae
- (C) INDIVIDUAL ISOLATE: Ljungan 174F

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGTCTAGTTT CATTCTGTGT GTGTTGGCA CTGAAATTAT TTCTGTCTCT GGGTGCTTT	60
ACACCTCAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC ACAGGAATTG CACAGGTGGC	120
TTTCACCTCT GGACAGTGCA TTCCACACCC GCTCCACAGT AGAAGATGAT GTGTGTCTTT	180
GCTTGTGAAA AGCTTGTGAA AATCGTGTGT AGGCGTAGCG GTACTTGAGT GCCAGCGGAC	240
ACCCCTAGTG GTAACACTAG C	261

## 2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 264 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Picornaviridae
- (C) INDIVIDUAL ISOLATE: Ljungan 145SL

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGTTTGGTTC TCTCTTGAGT GTGTTTGAGT TTAGCATAAT TTCTGTCTCT AGAGTGCTTT	60
ACACTCTAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC ACAGGAATCT GCACAGGTG	120
CTTTCACCTC TGGACAGTGC ATTCCATACC CGCTCCACAA TAGAAGATGA TGTATATCTT	180
TGTTTGTGAA ATGCTCATGA AACGTGTGTG TAGGCGTAGC GGCTACTTGA ATGCCAGCGG	240
AACCCCCCTA GTGGTAACAC TAGC	264

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 179 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (iii) HYPOTHETICAL: NO

## (v) FRAGMENT TYPE: internal

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Picornaviridae
- (C) INDIVIDUAL ISOLATE: Ljungan 145SL

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Lys Asp Leu Met Glu Ile Ala Arg Met Pro Ser Val Tyr Lys Gly Glu  
1 5 10 15

Arg Thr Glu Pro Gly Gly Thr Asn Gly Tyr Phe Gln Trp Ser His Thr  
20 25 30

His Ser Pro Ile Asn Trp Val Phe Asp Gly Gly Ile His Leu Glu Asp  
35 40 45

Met Pro Asn Leu Asn Leu Phe Ser Ser Cys Tyr Asn Tyr Trp Arg Gly  
50 55 60

Ser Thr Val Leu Lys Leu Thr Val Tyr Ala Ser Thr Phe Asn Lys Gly  
65 70 75 80

Arg Leu Arg Met Ala Phe Phe Pro Ile Met Met Gln Gly Thr Gln Arg  
85 90 95

Lys Lys His Lys Cys Leu Phe Met Val Cys Asp Ile Gly Leu Asn Asn  
100 105 110

Thr Phe Glu Met Thr Ile Pro Tyr Thr Trp Gly Asn Trp Met Arg Pro  
115 120 125

Thr Arg Gly Ser Val Ile Gly Trp Leu Arg Ile Asp Val Leu Asn Arg  
130 135 140

Leu Thr Tyr Asn Ser Ser Pro Asn Ala Val Asn Cys Ile Leu Gln  
145 150 155 160

Val Lys Met Gly Asn Asp Ala Lys Phe Met Val Pro Thr Thr Ser Asn  
165 170 175

Ile Val Trp

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**CLAIMS**

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1. Ljungan picornavirus, comprising in the non-coding region of its viral genome a nucleotide sequence corresponding to a cDNA sequence selected from the group consisting of

10 SEQ ID NO: 1 (Ljungan 87-012)

AGTCTAGTCT TATCTTGAT GTGTCCTGCA CTGAACTTGT TTCTGTCTCT 50  
GGAGTGCTCT ACACCTCAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC 100  
ACAGGAATCT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCACACC 150  
CGCTCCACCG TAGAAGATGA TGTGTGTCTT TGCTTGTGAA AAGCTTGTA 200  
AAATCGTGTG TAGGCGTAGC GGCTACTTGA GTGCCAGCGG ATTACCCCTA 250  
GTGGTAACAC TAGC

15 and homologous sequences having at least 75% homology to the SEQ ID  
20 NO: 1,  
and further, causing mammalian disease.

25 2. Ljungan picornavirus according to claim 1, wherein said homologous sequences have at least 80%, at least 85% or at least 90% homology to the SEQ ID NO: 1.

30 3. Ljungan picornavirus according to claim 2, wherein said homologous sequence is one of

35 SEQ ID NO:2 (Ljungan 174F)

AGTCTAGTTT CATTCTGTGT GTGTTGGCA CTGAAATTAT TTCTGTCTCT 50  
GGGGTGCTTT ACACCTCAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC 100  
ACAGGAATNT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCACACC 150  
CGCTCCACAG TAGAAGATGA TGTGTGTCTT TGCTTGTGAA AAGCTTGTA 200  
AAATCGTGTG TAGGCGTAGC GGNTACTTGA GTGCCAGCGG ACNACCCCTA 250  
GTGGTAACAC TAGC

35 and  
40 SEQ ID NO:3 (Ljungan 145SL).

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AGTTTGGTTCTCTCTTGAGTGTGTTTGTTAGCATAATTCTGTCTCT 50  
AGAGTGCTTTACACTCTAGTAGGGGCTGTA CCCGGCGGT CCCACTCTTC 100  
ACAGGAATCTGCACAGGTGGCTTACACCTCTGGACAGTGC ATTCCATACC 150  
CGCTCCACAA TAGAAGATGATGTATATCTT TGTTTGAA ATGCTCATGA 200  
5 AACGTGTGTG TAGGCGTAGC GGCTACTTGA ATGCCAGCGGAACCCCCCTA 250  
GTGGTAACAC TAGC.

4. Protein comprising an amino acid sequence selected from the group  
10 consisting of

SEQ ID NO: 4 ( partial structural protein of Ljungan 145SL)

Lys Asp Leu Met Glu Ile Ala Arg Met Pro Ser Val Tyr Lys Gly Glu  
5 10 15  
15 Arg Thr Glu Pro Gly Gly Thr Asn Gly Tyr Phe Gln Trp Ser His Thr  
20 25 30  
His Ser Pro Ile Asn Trp Val Phe Asp Gly Gly Ile His Leu Glu Asp  
35 40 45  
Met Pro Asn Leu Asn Leu Phe Ser Ser Cys Tyr Asn Tyr Trp Arg Gly  
20 50 55 60  
Ser Thr Val Leu Lys Leu Thr Val Tyr Ala Ser Thr Phe Asn Lys Gly  
65 70 75 80  
Arg Leu Arg Met Ala Phe Phe Pro Ile Met Met Gln Gly Thr Gln Arg  
85 90 95  
25 Lys Lys His Lys Cys Leu Phe Met Val Cys Asp Ile Gly Leu Asn Asn  
100 105 110  
Thr Phe Glu Met Thr Ile Pro Tyr Thr Trp Gly Asn Trp Met Arg Pro  
115 120 125  
Thr Arg Gly Ser Val Ile Gly Trp Leu Arg Ile Asp Val Leu Asn Arg  
30 130 135 140  
Leu Thr Tyr Asn Ser Ser Pro Asn Ala Val Asn Cys Ile Leu Gln  
145 150 : 155 160  
Val Lys Met Gly Asn Asp Ala Lys Phe Met Val Pro Thr Thr Ser Asn  
165 170 175  
35 Ile Val Trp ,

and homologous sequences having at least 75% homology to the SEQ ID  
NO: 4,  
and antigenic fragments of the sequences.

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30

5. Antiserum or antibody directed against a structural protein of the virus according to any one of claims 1-3.
- 5      6. Antigen comprising at least a part of a structural protein of the picornavirus according to any one of claims 1-3.
- 10     7. Diagnostic kit comprising at least one member from the group consisting of an antiserum or antibody according to claim 5 or an antigen-binding part thereof, an antigen according to claim 6 or an antibody-binding part thereof, one or several probes designed with respect to the genome of the virus according to any one of claims 1-3, and one or several primers designed with respect to the genome of the virus according to any one of claims 1-3.
- 15     8. Vaccine having as an immunizing or neutralizing component a member selected from the group consisting of a) the virus according to any one of claims 1-3, b) the virus according to any one of claims 1-3 in attenuated form, c) the virus according to any one of claims 1-3 in killed form, d) an antigen according to claim 6, including a subunit of the virus according to any one of claims 1-3, and e) DNA corresponding to the genomic RNA of the virus according to any one of claims 1-3.
- 25     9. Vaccine according to claim 8 which additionally comprises an adjuvant.
- 30     10. Ljungan picornavirus according to any one of the claims 1-3, optionally in attenuated or killed form, an antiserum or antibody according to claim 5 or an antigen according to claim 6 for use in a medicament.

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11. Use of a Ljungan picornavirus according to any one of the claims 1-3, optionally in attenuated or killed form, an antiserum or antibody according to claim 5 or an antigen according to claim 6, in the preparation of a medicament for prophylactic or therapeutic treatment of a disease caused by said virus.

5

12. Use according to claim 11, wherein the disease caused by said virus is one of Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome.

10

13. Method of prophylactic or therapeutic treatment of a disease caused by a virus according to any one of the claims 1-3 in a mammal, including human, which comprises administering to said mammal a prophylactically or

15

therapeutically effective amount of a medicament comprising as an active ingredient a member of the group consisting of

a) the virus according to any one of claims 1-3,

b) the virus according to any one of claims 1-3 in attenuated form,

c) the virus according to any one of claims 1-3 in killed form,

20

d) an antigen according to claim 6, including a subunit of the virus according to any one of claims 1-3,

and

e) DNA corresponding to the genomic RNA of the virus according to any one of claims 1-3.

25

14. Method according to claim 13, wherein the disease caused by said virus is one of Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome.

30

ATTORNEY/DOCKET No: REF/NIKLASSON/29713

**DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY**

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention (Design, if applicable) entitled: **NEW PICORNAVIRUSES, VACCINES AND DIAGNOSTIC KITS**

the specification of which (check one):

is attached hereto, or  was filed on: **September 9, 1997** as U.S. Application Number or PCT International Application Number: **PCT/SE97/01515** and (if applicable) was amended on:

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in *Title 37, Code of Federal Regulations, §1.56*. I hereby claim foreign priority benefits under *Title 35, United States Code §119* of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)			PRIORITY CLAIMED	
Number	Country	Day/Month/Year Filed	Yes	No
9603305-5	SE	11 September 1996	X	

 Additional Priority Application(s) Listed on Following Page(s)

I HEREBY CLAIM THE BENEFIT UNDER TITLE 35 U.S. CODE §119(E) OF ANY U.S. PROVISIONAL APPLICATIONS LISTED BELOW.	
Application Number	Day/Month/Year Filed

 Additional Provisional Application(s) Listed on Following Page(s)

I hereby claim the benefit under *Title 35, United States Code, §120* of any United States application(s) or PCT international application(s) designating The United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of *Title 35, United States Code, §112*, I acknowledge the duty to disclose information which is material to patentability as defined in *Title 37, Code of Federal Regulations, §1.56* which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application.

Application Number	Filing Date	Status - Patented, Pending or Abandoned

 Additional US/PCT Priority Application(s) listed on Following Page(s)

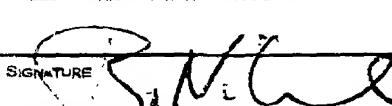
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under *section 1001 of title 18 of the United States Code* and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: J. Ernest Kenney, Reg. No. 19,179; Eugene Mar, Reg. No. 25,893; Richard E. Fichter, Reg. No. 26,382; Charles R. Wolfe, Jr., Reg. No. 28,680; Thomas J. Moore, Reg. No. 28,974; Joseph DeBenedictis, Reg. No. 28,502; Benjamin E. Urias, Reg. No. 33,805; and

I (we) authorize my(our) attorneys to accept and follow instructions from Stockholms Patentbyrå regarding any matter related to the preparation, examination, grant and maintenance of this application, any continuation, continuation-in-part or divisional based thereon, and any patent resulting therefrom, until I(we) or my(our) assigns withdraw this authorization in writing.

Send correspondence to: BACON & THOMAS  
625 Slaters Lane - 4th Floor  
Alexandria, VA 22314-1176

Telephone Calls to: (703) 683-0500

FULL NAME OF FIRST OR SOLE INVENTOR <b>Bo NIKLASSON</b>	CITIZENSHIP <b>Sweden</b>
RESIDENCE ADDRESS <b>Sibyllegatan 15 S-114 42 Stockholm, SWEDEN</b>	POST OFFICE ADDRESS IS THE SAME AS RESIDENCE ADDRESS UNLESS OTHERWISE SHOWN BELOW
DATE <b>March 5 1995</b>	SIGNATURE 

 See following page(s) for additional joint inventors.